EMBO reports

Manuscript EMBO-2017-44000

TGFβ1-induced leucine limitation uncovered by differential ribosome codon reading

Fabricio Loayza-Puch, Koos Rooijers, Jelle Zijlstra, Behzad Moumbeini, Esther A. Zaal, Joachim F. Oude Vrielink, Rui Lopes, Alejandro Pineiro Ugalde, Celia R. Berkers, and Reuven Agami

Corresponding author: Fabricio Loayza-Puch and Reuven Agami, The Netherlands Cancer Institute

Review timeline:

Submission and transfer date:	26 January 2017
Editorial Decision:	01 February 2017
Revision received:	03 February 2017
Accepted:	08 February 2017

Editor: Esther Schnapp

Transaction Report: This manuscript was transferred from *The EMBO Journal*, where it was originally reviewed. The following report contains those referee comments that were outstanding at the time of transfer.

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 February 2017

Thank you for the transfer of your revised manuscript to EMBO reports. I have now heard back from referee 2 who supports the publication of your revised study here, after careful re-writing of the manuscript text. Please focus on the inhibitory effect of TGFb on cell proliferation instead of on EMT, which is still mentioned in several places, including the abstract. I would also like to suggest to change the title in order to include the main findings. Please also add up to 5 keywords.

Please clarify in the figure legends whether the statistical info refers to all or only some figure panels, as this is unclear in several cases. Figure EV4 is missing this info. Figure EV1 is uploaded at insufficient quality, and figure EV2 might need panel letters.

You can either publish your manuscript as a short report (with a max of 5 figures and a combined results and discussion section), or as a full-length article (with at least 6 figures and separate results and discussion sections).

Please change the reference style into the numbered EMBO reports style that can be found in EndNote.

We have a suggestion from you for the short summary and bullet points, but we are still missing a synopsis image for our website. The image should be 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us the image along with the revised manuscript.

****
PRIOR REFEREE REPORTS

Referee #1:

The authors have adequately addressed the previous concerns raised in the initial critique, particularly the EMT points. Since the manuscript have been re-routed, the authors should de-emphasize most of the introductory points related to this process

Referee #2:

The revised paper by Loyaza-Puch utilized differential ribosome codon reading (diricore) technology to analyze the response of breast epithelial cells MCF10A to the cytokine TGF-beta1. The MCF10A cell model and TGF-beta1 were used based on interest to analyze the differential codon use by ribosomes, during the process of epithelial-mesenchymal transition (EMT). Diricore relealed a relative deficiency in the amino acid leucine pool in the cells that were treated with TGF-beta1. This deficiency translated to a deficiency in Leu-tRNA loading and was explained by a negative effect of TGF-beta1 on the expression level of SLC3A2, a plasma membrane leucine transporter. TGF-beta1 not only induced EMT of the breast cells, but also reduced the rate of their proliferation. The reduction in Leu levels has therefore been linked to this cellular response.

This reviewer and reviewer 1 previously requested stronger evidence for a functional link between Leu pool deficiency, SLC3A2 level decrease and EMT in the MCF10A cell model. The experimental evidence provided by the authors failed to make this link and for this reason the title of the paper has been changed and the final conclusions have been changed somewhat to reflect this deficiency. Despite the implemented changes, the paper is heavily focused on EMT, at least in the abstract, synopsis, introduction and discussion. Thus, although the authors have formally "responded" to the reviewers comments they want to deliver a scientific article that pushes the reader to learn about how a decrease in Leu availability impacts the EMT, and in the end they do not do this. Even if one takes a different view of the matter, i.e. TGF-beta1 signaling and the mechanism of cell cycle arrest, a topic that is heavily studied and very rich in literature, this topic is not either properly addressed. The introduction does not touch upon this topic and thus does not explain how TGF-beta1 signaling causes epithelial cell cycle arrest. The single experiment of Figure 3E that shows that exogenous Leu can partially counteract the reduced proliferation of cells stimulated with TGF-beta1, does not really explain any novel mechanism that establishes how deficiency in an amino acid pool contributes to cell cycle arrest. Neither is the contribution of the SLC3A2 transporter to this process if analyzed.

In conclusion this paper analyzes the possibility that TGF-beta1 signaling might impact on differential ribosome codon reading and it provides a positive response: Leu codons are indeed differentially read. And then the paper explains that this is due to a decrease in leu, which is due to a decrease in a SLCR3A2 expression. The authors claim that the latter is a direct and rapid response to TGF-beta signaling, but no experimental evidence attempts to query this aspect.

This is the second time I review this paper. I do not support its publication as the paper currently stands. For the interests of the authors and the journal that wants to publish this paper, I suggest that the journal identifies a new reviewer that can provide an independent point of view.

My personal specific comments this time are as follows:
1. The new title accurately reflects the content and major conclusion of the paper.
2. The rest of the paper needs to be built along the lines guided by the title.
3. This means that the abstract can be re-written to de-emphasize EMT, metastasis and chemoresistance. None of these relate to the content of the paper.
4. The synopsis must be re-written in the same spirit.
5. The introduction must be re-written extensively. The details on ZEB1 are not relevant. The link of EMT to metabolism and chemoresistance are not relevant. Some introduction about cell proliferation and its control by TGF-beta1 would be useful and relevant.
6. The results are to the largest extent fine. I would advise for the sake of non-specialists that all figures that present tRNA-amino acid loading experiments (Figure 2C, 2D, 3A, 4D, 5C) include in

their legends the detail that amino acid-anti-codon (and not codon) are shown. This helps as many other figures refer to codons. EV Figure 1 is unrelated to the paper, it can be removed. Figure 3E can be complemented with siSLC3A2 and SLC3A2 overexpression analyses on cell proliferation. EV Figure 5B can be enhanced with more early time points and cycloheximide experiments, knockdown of Smad proteins to establish direct and rapid transcriptional regulation of SLC3A2 by TGF-beta1
7. The Discussion has to be re-written to de-emphasize EMT (first long paragraph) and emphasize cell proliferation. The discussion on SLC3A2 and YAP/TAZ is not clear. The statement "TGF-beta1 was already connected to YAP/TAZ signaling..." is vague. The exact connection should be presented and explanations on how this fits with the current evidence that TGF-beta1 downregulates SLC3A2. A Reference to the YAP/TAZ-TGF-beta link is missing. Finally, discussion on the impact of limited ribosomal translation and cell cycle arrest by TGF-beta can be included in the discussion. Specific mechanistic facts may be used instead of general statements that make no sense.

1st Revision - authors' response 03 February 2017

Thanks, once more, for accepting our paper. As you can imagine, we were delighted to hear about your decision. I have now uploaded the final version to your website.

In the final version of the manuscript, we addressed all your comments.

We removed some mentions to EMT in the abstract and text, but still some remain in the introduction and discussion (these are essential).

We clarified the statistical information in all figures, and increased as much as possible the resolution of fig EV1 and added panels to Fig EV2

We also include a synopsis image + text.

Finally also the references were adapted to your style.

2nd Editorial Decision 08 February 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Reuven Agami
Journal Submitted to:	EMBO
Manuscript Number:	EMBOJ-2016-95445

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Not applicable to our studies
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	all samples of the of the experiments were included
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not applicable to our studies
For animal studies, include a statement about randomization even if no randomization was used.	not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Not applicable to our studies
4.b. For animal studies, include a statement about blinding even if no blinding was done	not applicable
5. For every figure, are statistical tests justified as appropriate?	Yes in every legend to figure the statistical tests are justified
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, the data meet the assumptions of the tests
Is there an estimate of variation within each group of data?	depending on the statisticak analysis we estimated the variation within each group of data
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	okay, see methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	yes, see methods

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	not applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	yes, we provided
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	okay
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	okay
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	okay
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	not applicable

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no